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Author Contributions

HF & DHN designed the study and wrote the first draft of the manuscript; JMP & JGP conducted fieldwork and managed the long-term study; SLU, JD, LAS, KW & RVW conducted the labwork; HF conducted the analyses; all authors contributed to the revised manuscript.

This PDF file includes: Main Text; Figures 1 to 3.

Abstract

Telomere length (TL) is considered an important biomarker of whole-organism health and ageing. Across humans and other vertebrates, short telomeres are associated with increased subsequent mortality risk, but the processes responsible for this correlation remain uncertain. A key unanswered question is whether TL–mortality associations arise due to positive effects of genes or early-life environment on both an individual's average lifetime TL and their longevity, or due to more immediate effects of environmental stressors on within-individual TL loss and increased mortality risk. Addressing this question requires longitudinal TL and life-history data across the entire lifetimes of many individuals, which are difficult to obtain for long-lived species like humans. Using longitudinal data and samples collected over nearly two decades, as part of a long-term study of wild Soay sheep, we dissected an observed positive association between TL and subsequent survival using multivariate quantitative genetic models. We found no evidence that telomere attrition was associated with increased mortality risk, suggesting that TL is not an important marker of biological ageing or exposure to environmental stress in our study system. Instead, we find that among-individual differences in average TL are associated with increased lifespan. Our analyses suggest that this correlation between an individual's average TL and lifespan has a genetic basis. This demonstrates that TL has the potential to evolve under natural conditions, and suggests an important role of genetics underlying the widespread observation that short telomeres predict mortality.

Significance Statement

Telomeres play an important role in ageing and having relatively short telomeres is associated with an increased risk of death in humans and other animals. Telomere length is influenced by both genetic and environmental factors, both of which could potentially drive the positive association with survival. We used lifelong telomere length measurements from a population of wild sheep to disentangle this relationship. For the first time in a natural population, our analyses reveal a genetic correlation between telomere length and longevity but no association between telomere shortening and mortality risk. These findings have important implications for our understanding of telomere dynamics and their role in health and lifespan.

Main text

Introduction

Telomeres are repetitive sequences of non-coding DNA found at the terminal ends of linear chromosomes, and they play an important role in maintaining DNA stability and integrity (1-3). Telomeres shorten during cell replication and in response to oxidative stress (4, 5), and cellular senescence and apoptosis is triggered once telomeres reach a critically short threshold (2). The important role of telomeres in cellular senescence has led to telomere shortening being considered as one of nine “hallmarks of ageing”, and average telomere length (TL) as an important biomarker of whole-organism health and biological ageing (6). In humans, relatively short leukocyte telomeres have been linked to a range of age-related diseases such as diabetes, cancer and cardiovascular disease (7-9) and increased subsequent mortality risk (10-12). A recent meta-analysis suggests this pattern may generalise beyond humans: across studies from 20 non-model vertebrate species (predominantly birds) there was an overall positive association between telomere length and subsequent survival (13). Although evidence for a causal role for telomeres in whole-organism ageing and longevity remains weak (14), these findings highlight the potential significance of TL as a biomarker of human and animal health (15, 16) and for our understanding of life-history evolution (17, 18).

Studies in humans and other vertebrates have found evidence for consistent differences in TL among individuals over multiple measurements (19, 20). Such repeatable among-individual differences in any trait may result from the trait being under genetic influence, from long-term effects of the early-life environment, and/or environmental conditions that persist across the lifetime. There is good evidence that variation in average TL in blood cells has a genetic basis in humans and other vertebrates, although estimates of the heritability (the proportion of variation attributed to additive genetic effects) of TL are variable (21, 22). Recent studies of wild vertebrates have also revealed considerable variation in adult TL among birth cohorts, suggesting persistent impacts of early-life environment (23, 24). At the same time, there is growing evidence that TL is highly dynamic across an individual’s lifetime, and meta-analyses of human and non-human animal studies show that experience of diverse forms of environmental stress are predictive of shorter TL (25-27). Indeed, some studies using longitudinal TL data have found that telomere shortening over successive measurements rather than telomere length *per se* is predictive of mortality (28-30). Thus, the emerging picture from studies in humans and other vertebrates is that shorter TL generally

predicts increased risk of subsequent mortality, and that variation in TL is under the influence of both genetics and environmental stressors.

The observation that shorter TL measurements predict increased mortality risk could be underpinned by two, non-mutually exclusive processes operating across the lifetimes of individuals. Firstly, individuals may differ in their average TL across life, and individuals with shorter TL may be shorter lived. This pattern is referred to as the 'selective disappearance' of individuals with shorter telomeres, and it implies that TL reflects constitutive differences among individuals (for example due to genetics or differences in early-life environment) which shape their longevity (31, 32). Secondly, individuals may differ in their pattern of TL change over time, and individuals showing the greatest telomere loss across successive measurements are more likely to die subsequently. This pattern is consistent with the idea that within-individual telomere dynamics reflect recent and cumulative experiences of environmental stress and physiological deterioration that also predict mortality. Neither pattern necessarily implies a causal role for telomeres in driving the mortality risk of an organism, because associations between TL and survival could result from both traits being correlated with underlying, unmeasured variables which causally impact survival (14, 18). Nevertheless, unravelling the contribution of genetics, early-life environment and more immediate telomere shortening to the observed association between TL and survival is essential for our understanding of TL as a biomarker of health and ageing (19).

To our knowledge, no study to date has assessed the relative importance of the different processes underlying the relationship between TL and mortality risk across the entire lifespan. To do so demands repeated measurements from across life to characterise among- and within-individual variation in telomere length, a population pedigree or genomic information to separate genetic and environmental sources of variation, and detailed information on individual health and fitness outcomes over the lifetime. Here, we use a multivariate mixed-effects modelling approach to analyse extensive, longitudinal data from a long-term study of wild Soay sheep living on St Kilda, Scotland, to distinguish between possible models of *why* shorter TL predicts increased mortality risk. We find that the observed positive association between TL and mortality in this system is underpinned by selective disappearance of individuals with shorter average TL. Importantly, our results suggest this is largely driven by genetically-based differences in both TL and longevity.

Results

Soay sheep resident to our study area on St Kilda have been individually marked at birth and closely monitored and repeatedly blood sampled across their lifetimes. Here, we measured relative telomere length (RTL) in 3641 samples collected from 1586 individual sheep over a 19-year period (see Methods). We found that RTL declined with age in Soay sheep, with a more rapid initial decline between measurements at around 4 and 16 months, followed by a slower linear decline thereafter (Figure 1). The best fitting age function in our models of RTL included a two-level factor for age class (lambs and adults aged ≥1 year) and a linear term for age in years, which is equivalent to a segmented regression with a threshold at one year of age (SI Appendix, Tables S1 & S2). There was considerable variation in RTL within age groups (SI Appendix, Fig. S1). There was limited evidence for a sex difference in either average RTL or the rate of change in RTL with age, although the significance of sex in our model depended on model structure (see SI Appendix). The individual repeatability of RTL over the lifespan was 0.214 (95%CI 0.169–0.252, SI Appendix, Table S2). Excluding the variance attributed to qPCR plate and row (which represents measurement error) from the denominator, the repeatability was 0.241 (95%CI 0.204–0.282, SI Appendix, Table S2). Although RTL declined with age on average, we found evidence for consistent differences in RTL among individuals.

There was detectable additive genetic variance for relative telomere length across all ages in the population (Figure 2; SI Appendix, Table S3). The heritability of telomere length (the proportion of variance explained by additive genetic effects) was 0.204 (95%CI: 0.158–0.252). The permanent environment effect was bound at zero (<0.001, 95%CI: <0.001–0.017), indicating that individual repeatability in telomere length could be almost entirely be attributed to genetic rather than environmental effects. Maternal effects also explained a very small proportion of the variance (<0.001, 95%CI <0.001–0.034, estimate bound at zero). The year the sample was collected, qPCR plate and qPCR row each explained 3-4% of the variance in telomere length (year: 0.031, 95%CI 0.014–0.084; qPCR plate: 0.043, 0.027–0.066; qPCR row: 0.038, 0.014–0.172). Excluding the measurement error terms of qPCR plate and row from the total phenotypic variance, the heritability of telomere length was 0.233 (95%CI 0.189–0.279). This shows that variation in telomere length has a genetic basis in wild Soay sheep.

We went on to estimate the genetic correlation between RTL expressed in lambs, which are still developing at the time of measurement (aged 4 months), and in older individuals, which are sexually mature and have largely completed growth. When estimated with a bivariate model of lamb and adult telomere length, the heritability of telomere length in lambs was estimated to be 0.285 (95%CI 0.206–0.369), and in adults 0.210 (95%CI: 0.156–0.255)

(Figure 2; SI Appendix, Table S4). The genetic correlation between lambs and adults was close to 1 (0.916, 95%CI: 0.806–0.996, SI Appendix, Table S4), implying that largely the same or linked genes influenced RTL across age groups. The residual correlation between lamb and adult telomere length was close to zero (0.036, 95% CI: -0.196–0.008, SI Appendix, Table S4). The negligible residual correlation was expected given the lack of permanent environment effect underlying repeatable differences in individual RTL across all ages (SI Appendix, Table S3). Repeatable among-individual differences across ages in RTL were therefore driven predominantly by genetic rather than environmental effects, and a similar set of genes influenced RTL across ages.

Using multivariate mixed-effects models, we next tested the strength and direction of correlations among RTL, body weight (both measured in August) and subsequent overwinter survival at different hierarchical levels (among-individual, genetic, among-year, within-individual). Although we were primarily interested in the RTL–survival association, the inclusion of weight in our models allowed us to contrast the magnitude of the RTL–survival association with a well-studied trait which is known to be linked to condition and fitness in our study system (33). As previously documented, August body weight was strongly predictive of improved winter survival prospects ((33-35); Figure 3). Our multivariate models revealed that this association was present at all hierarchical levels (Figure 3; SI Appendix, Tables S5 & S6). Our initial phenotypic model indicated that heavier individuals on average tended to have longer lifespans (Figure 3A; SI Appendix, Tables S5). Developing this model into a pedigree-based quantitative genetic model revealed that this among-individual effect was driven by similar sized contributions from genes and environment (Figure 3B; SI Appendix, Table S6). Overwinter survival probabilities were higher in years where the average body weight was higher (among-year effect), and individuals with relatively low weight compared to their average weight were less likely to survive (residual effect; Figure 3). In our phenotypic model, there was little evidence for an association between RTL and body weight at any hierarchical level (Figure 3A; SI Appendix, Table S5). However, the quantitative genetic model revealed a negative genetic correlation (Figure 3B; SI Appendix, Table S6). This was consistent in separate models of lambs and adults, although here credible intervals overlapped zero more widely (SI Appendix, Tables S7-S10).

We found a positive association between telomere length and overwinter survival probability at the among-individual level (Figure 3A; SI Appendix, Table S5). This suggests that individuals with longer telomeres on average across their lives tended to have longer lifespans. The 95% credible intervals overlapped zero for both the among-year and the residual covariance between telomere length and overwinter survival (Figure 3A; SI

Appendix, Table S5). The absence of an association at the within-individual (or residual) level indicates that if an individual had a relatively short RTL measurement compared to their average in a particular year, this was not associated with an increased risk of mortality. Overall, these findings imply that average telomere length after weaning, rather than more immediate changes relative to an individual's average TL, predicted overwinter survival.

The quantitative genetic model revealed a positive association between telomere length and overwinter survival probability at the genetic level, though the credible intervals narrowly overlapped zero (Figure 3B; SI Appendix, Table S6). The covariance between TL and survival was very small (<0.001) at the permanent environment level (SI Appendix, Table S6) and the correlation at this level had wide credible intervals which extensively crossed zero (Figure 3B). Our results were broadly consistent for lambs and adults across separate models, although the credible intervals of the among-individual and genetic effects did narrowly cross zero within some age groups (Figures S2 & S3; SI Appendix, methods & results, Tables S7-10). Overall, our results suggest that the among-individual positive association between telomere length and survival identified in the phenotypic model was driven largely by genetic effects (Figure 3B; SI Appendix, Table S6). In other words, genes conferring longer average telomeres after weaning also tended to be associated with longer lifespan.

Discussion

A growing number of studies across vertebrate species have shown that TL is heritable (21) and that relatively short TL is predictive of increased mortality risk (13). We have dissected the TL–mortality relationship across the natural lifespan of wild Soay sheep to identify the processes that drive this association. We show that selective disappearance of individuals with shorter average TL is the key process, rather than an association between the pattern of TL attrition and mortality. If TL was a marker of biological ageing, or it reflected variation in condition resulting from environmental stress, we would expect telomere shortening to predict mortality, resulting in a positive residual correlation between TL and survival in our models. We found no support for a correlation at the residual level, and only the among-individual correlations between TL and survival were consistently different from zero. This suggests that TL is not a useful marker of biological ageing in our system. Furthermore, it is inconsistent with the hypothesis that TL reflects variation in physiological state or condition resulting from recent or accumulating experience of environmental stress during adulthood, which also influences mortality risk. However, our first TL measurement was taken at four months of age – around the time of weaning for most lambs in our study population – and it

231 is possible that associations between average TL and survival are driven by some
232 combination of initial TL and the rate of telomere attrition prior to first measurement. An
233 individual's average TL could therefore reflect its ability to maintain homeostasis and resist
234 environmental stress during very early development, which in turn could predict its
235 subsequent lifespan. Nonetheless, our results demonstrate that variation in TL is heritable
236 and under directional selection, meaning it has the potential to evolve under natural
237 conditions.

238 Our moderate estimate of the heritability of TL (around 20%, Figure 2) represents the first
239 evidence for an appreciable genetic contribution to variation in TL in a wild mammal. Recent
240 studies in wild badgers and bats have used similar pedigree-based 'animal models' but
241 found both the repeatability and heritability of TL to be negligible (36, 37). Our estimate was
242 lower than in a recent longitudinal study of farmed dairy cattle (32–38%; (38)), which is
243 unsurprising given the expectation of reduced environmental variation in livestock compared
244 to wild systems. It is notable that both studies found negligible permanent environment
245 effects, implying that consistent differences in TL across life were largely the result of genetic
246 rather than early environmental effects in both systems ((38); Figure 2). More broadly, while
247 human studies have tended to find moderate to high heritability of TL, the growing literature
248 in birds presents a much more variable picture (21). Two laboratory studies of birds have
249 estimated the heritability of TL to be >100% (39, 40), whilst some studies in the wild suggest
250 little genetic influence on TL (heritability <5% (41, 42)). The reasons for the variation in
251 heritability estimates across studies remain an important area for future research. As well as
252 variation in the way that genes influence telomere dynamics, differences in the
253 environmental variation experienced by different populations or species, and the degree of
254 error associated with different telomere measurement methodologies are also likely to play a
255 role (21). Our study used a qPCR method to measure TL, which is often described as having
256 greater measurement error than the 'gold standard' terminal restriction fragment (TRF)
257 approach (43). However, we note that the technical repeatability of our qPCR assay is high
258 and compares favorably to those reported for TRF studies (see Methods). Furthermore,
259 most previous studies in humans and birds have focused on specific age classes (e.g.
260 elderly humans or pre-fledging birds). Our data spans the entire lifetimes of individuals, and
261 we were able to demonstrate a very high genetic correlation between TL measured in lambs
262 and adults. This provides clear evidence that the same or linked genes influence TL in early
263 and later life, an assumption that has rarely been tested in any study system (though see
264 (44)).

In a previous study, we identified an association between TL and mortality in a much smaller sample of female Soay sheep (45). In that study, we reported a considerably lower repeatability for TL (13%) than in the present study and were only able to detect a TL–mortality relationship among younger females experiencing a high mortality winter. This previous study was focused on a subset of females from four birth cohorts, and thus had relatively few samples from later adulthood (45). Our present study is distinguished by the much larger data set and more complete population coverage, which has allowed us to assess the repeatability of TL and determine that it is the among-individual differences in TL across life that are predictive of mortality. We note that, when splitting our multivariate models by age groups, the among-individual covariance between TL and survival was positive and of a similar magnitude in adults and lambs, though the credible intervals overlapped zero (SI Appendix, Figs. S2 & S3; Table S4). This indicates that the association was not simply due to effects on lamb survival. Although some longitudinal vertebrate studies have found that telomere attrition predicted survival better than average or recent TL (28, 29, 46), others have identified extremely high consistency in individual TL across measurements (19, 20) and long-term associations between early-life TL and adult lifespan (24, 47, 48). Most of these studies involved only one or two measurements of TL per individual, and none used a multivariate mixed-effects modelling approach capable of fully dissecting the contributions of genetic, individual, annual and residual sources to observed TL–survival covariance.

Recent studies of elderly human cohorts have identified candidate SNP loci associated with telomerase genes, which are involved in maintenance of telomeres and genomic integrity, that not only predict average leukocyte TL but also subsequent morbidity and mortality (49-51). However, human twin studies also suggest that relatively short TL, independent of genetic factors, predicts mortality (52). In support of a causal role for telomerase genetics in mortality and ageing, studies of laboratory mice without telomerase appear to show early onset of ageing phenotypes, while mice with genetically enlarged telomeres are longer lived (53, 54). Our findings provide the first support for a role for genetics in observed TL–mortality relationships from a non-human system outside the laboratory. Further work is required to determine whether specific genes, such as those involved in telomere maintenance, are implicated in this relationship or whether the observed genetic correlations result from minor effects of many different genes.

Our results imply that the observed relationship between TL and mortality is not causal. A causal effect of short telomeres on survival would lead to the expectation that both individuals with low average TL (among-individual covariance) and short TL at measurement

relative to their average (residual covariance) should be positively related to survival. But this is not what we observed. Instead, it seems likely that the genes influencing some yet to be determined aspect of an individual's overall frailty (or robustness) have a correlated influence on TL. These genes could be influencing TL determined during early embryonic development and/or the rate of telomere attrition during gestation and neonatal life, as our first TL measurement was not taken until around four months of age. However, comparing the magnitude of the TL–survival correlations with the correlations between body weight and survival highlights that the association between TL and mortality is modest compared to a measure that is more directly relatable to condition and health. It remains to be determined whether our findings in Soay sheep will generalize to other species and systems, but our work highlights the importance of using large data sets that span the entire lifetimes of many individuals, in order to fully understand the drivers of associations between TL, health and mortality risk. Overall, our results provide important insights into the genetics of lifespan in the wild and highlight the importance of long-term, longitudinal studies across different species for our understanding of TL as a biomarker of health and fitness.

Materials and methods

Study system and sample collection

The Soay sheep (*Ovis aries*) is a primitive breed of domestic sheep that has been living on the remote St Kilda archipelago with minimal human management for the last few millennia (57°49′N, 8°34′W; (33)). Since 1985, the sheep resident within the Village Bay area of the main island in the archipelago, Hirta, have been the subject of an individual-based study (33). Individuals are caught and tagged within a few days of birth in the spring. Ten censuses are conducted on each of three annual field seasons, during spring (March – April), summer (July – August) and autumn (October – November), meaning the timing of individual's disappearance is known with a high degree of accuracy (33). The vast majority of sheep mortality occurs in late winter (85% of adult deaths occur January – April), and daily carcass searches during this period mean that death dates are known to the nearest month for most individuals. Each August, 50–60% of the resident population are caught in temporary corral traps and blood sampled. Blood is collected from each individual into 9 ml lithium heparin Vacuettes and kept in a cool box or fridge from the point of sampling. The blood is processed within 24 hours to separate the plasma and buffy coat fractions. The Vacuette is then spun at 1,008 x g for 10 minutes, the plasma layer drawn off and replaced by the same quantity of 0.9% NaCl solution, gently mixed and spun again at 1,008 x g for 10 minutes. The intermediate buffy coat layer, comprising mainly white blood cells, is then

drawn off into a 1.5 ml Eppendorf tube and stored at -20 °C until used to assay leukocyte telomere length. All data collection was approved by the UK Home Office and carried out in accordance with the relevant guidelines.

Pedigree reconstruction

Parentage was inferred by genetic methods, except for some maternal links inferred by observation (55). Multi-generational pedigree reconstruction was performed in the R package *Sequoia* (56), using 431 unlinked SNP markers. This likelihood-based approach infers not only parent-offspring relationships, but also siblings and second-degree relatives. In the resulting pedigree, a mother and/or father was assigned to 6082 individuals. After pruning to only those individuals informative to the current analyses (using the R package *MasterBayes* (57)) , the pedigree had a maximum depth of 13 generations and consisted of 2411 individuals, of which 2273 were non-founders and a total of 2050 maternities and 2172 paternities were assigned.

Sample selection and randomization

A total of 3891 August buffy coat samples from 1647 animals of known age collected between 1998 and 2016 were selected for telomere length measurement. These samples were selected from the total available buffy coat freezer archive (n=6775 from 3315 sheep) based on the following exclusion criteria: samples collected before 1998 (n=1924); samples from individuals of unknown age (n=97); samples collected between 2013 and 2016 from lambs or yearlings that were caught only once (n=454); samples collected from individuals that were only captured once but survived, and were therefore available for sampling in subsequent years (n=409). These criteria were designed to maximise the longitudinality of the dataset while avoiding biasing the dataset against short-lived individuals (i.e. individuals only sampled once because they died). Sample years were then randomly allocated to one of four batches, each comprising 4–5 years (to reduce the number of samples that needed to be removed from the freezer at any one time). Samples were then fully randomised within each batch, assigned a unique identifier from the start of batch 1 to the end of batch 4, and processed from DNA extraction through to qPCR in this order.

DNA extraction

Genomic DNA was extracted from buffy coat on 96 well plates using the Macherey-Nagel Nucleospin 96 Blood kit (Cat# 740665). The samples were extracted on a liquid handling robot (Freedom Evo-2 150; Tecan) using a vacuum manifold. In order to facilitate passage of the sample through the DNA binding plate, the following step was included prior to automated extraction on the robot. 50 µl of buffy coat was mixed with 300 µl RBC lysis

solution (Qiagen; Cat# 158902) and incubated at room temperature for 5 minutes, before centrifugation at 12200 rpm for 30 seconds. 250 µl of the supernatant was discarded and the cell pellet was re-suspended in the residual supernatant, before it was transferred to a 96 well MN lysis block (Macherey-Nagel Nucleospin 96 Blood kit; Cat# 740665) and sealed. The liquid handling robot was loaded with the MN lysis block with the seal removed, the silica DNA binding plate, 100% Ethanol, lysis buffer BQ1, wash buffers B5 and BW and finally a master mix containing PBS, proteinase K and RNAse A (binding plate, buffers and proteinase K supplied with Macherey-Nagel Nucleospin 96 Blood kit, Cat# 740665; RNAse A, Qiagen Cat# 158924; PBS, Sigma Cat# D1408). The extraction protocol followed manufacturer's guidelines for use with a vacuum manifold with the following amendments. The robot added 96 µl 1X PBS, 25 µl Proteinase K and 4 µl RNAse A to each sample. Lysis was performed on a shaker for 10 minutes. The vacuum steps for binding and the first two washes were increased to 5 minutes and the final wash to 10 minutes with an additional 10-minute vacuum at the end to dry the membrane. If any lysate/wash failed to pass through the silica membrane after a vacuum step the plate was removed from the robot and centrifuged for 3 minutes at 4000 rpm. For the few samples that failed to pass though after centrifugation the wash/lysate was removed by hand with a pipette. After dry centrifugation (3 minutes, 4000 rpm), DNA was eluted in a total of 150 µl elution buffer BE (Macherey-Nagel Nucleospin 96 Blood kit; Cat# 740665) which was warmed to 60 °C prior to adding it onto the silica membrane. Elution was performed in two steps: first 100 µl buffer BE followed by centrifugation (3 minutes, 4000 rpm), then 50 µl buffer BE followed by a final centrifugation (3 minutes, 4000 rpm).

DNA extraction quality control

Following DNA extraction, a strict quality control protocol was implemented to measure DNA concentration, integrity and purity (SI Appendix, Figure S4). Of the 3891 samples selected for analysis, 42 samples were missing or accidentally omitted through human error. The available samples were measured on a Nanodrop ND-1000 9 spectrophotometer (Thermo Scientific, Wilmington DE, USA). Samples yielding < 20 ng/µl were rejected, and re-extraction attempted for 491 of these samples. Overall, 87 samples failed to yield sufficient DNA and were excluded from the analyses. Samples yielding ≥ 20 ng/µl were checked for DNA purity. The acceptable range for absorption for 260/280 nm ratio (a measure of DNA vs protein and RNA contamination) was 1.7–2.0. Samples falling outside of this range were excluded from subsequent analyses (n=66, representing a failure rate of 1.76%). Two thirds of samples were assayed for 260/230 nm ratio (a measure of salt and other impurities), and samples with a ratio <1.8 were excluded from subsequent analyses (n=41, representing a failure rate of 1.73%). Samples of sufficient yield and purity were standardized to 10 ng/µl

and DNA integrity was assessed by running 200 ng of DNA on a 0.5% agarose gel. Samples were scored for integrity on a scale of 1 to 5 by visual examination of their DNA crowns, and samples scoring 3 to 5 were excluded from further analyses (see (58)). Integrity was initially assayed for all of the first 1667 samples run. Only 16 of these samples were given a DNA integrity score of 2, and 7 failed (failure rate of 0.42%). We randomly tested a quarter of all subsequent samples for DNA integrity, with only 4 failing (representing a failure rate of 0.76%). In total, 11 samples were excluded due to poor DNA integrity. Overall, DNA was successfully extracted and passed quality control requirements from 3644 buffy coat samples (SI Appendix, Figure S4).

Telomere length measurement

Quantitative polymerase chain reaction

Relative leukocyte telomere length (RTL) was measured using real-time quantitative PCR (qPCR; (59)), using protocols we have previously developed and validated using blood samples from sheep and cattle (45, 58). The qPCR method estimates the total amount of telomeric sequence present in a sample relative to the amount of a non-variable copy number reference gene. In this study we used the beta-2-microglobulin (B2M) as our reference gene (45, 58), with primers supplied by Primer Design (Cat# HK-SY-Sh-900, Southampton, UK). For telomeric amplification tel1b (5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3') and tel 2b (5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3') primers were used. Telomere primers were manufactured, HPLC purified and supplied by Integrated DNA Technologies (IDT, Glasgow, UK). Telomere and reference gene reactions were run in separate wells of the same qPCR plate at a concentration of 300 nM and 900 nM, respectively. Samples were diluted to 0.5 ng/µl with buffer BE (Macherey-Nagel Nucleospin 96 Blood kit; Cat# 740665) immediately prior to qPCR analysis. Each reaction was prepared using 5 µl of LightCycler 480SYBR Green I Master Mix (Cat # 04887352001, Roche, West Sussex, UK) and 1 ng of sample DNA in a total reaction volume of 10 µl. We used 384 well plates which were loaded with sample DNA and master mix using an automated liquid handling robot (Freedom Evo-2 150; Tecan).

Each plate included 8 calibrator samples (1 ng/µl) to account for plate to plate variation and two non-template controls (NTC) consisting of nuclease free water. The calibrator sample was extracted from a large quantity of buffy coat prepared from blood supplied from a single domestic sheep (Cat# SHP-BUFCT-LIHP, Sera Laboratories International LTD, West Sussex, UK). We carried out a large number of extractions from this sample, applied the same quality control as above and then pooled the extracts and aliquoted them for subsequent use. A five-point standard curve, consisting of a four-fold serial dilution of the

calibrator sample (at concentrations: 20 ng/µl, 5ng/µl, 1.25 ng/µl, 0.3125 ng/µl and 0.078125 ng/µl) was included on the plate to provide a visual check that the samples amplified at the correct cycle. Samples, calibrators, standard curve and NTCs were all run in triplicate. All qPCRs were performed using a Roche LC480 instrument using the following reaction protocol; 10 min at 95 °C (enzyme activation), followed by 50 cycles of 15 s at 95 °C (denaturation) and 30 s at 58 °C (primer annealing), then 30 s at 72 °C (signal acquisition). Melting curve protocol was 1 min at 95 °C, followed by 30 s at 58 °C, then 0.11 °C/s increase to 95 °C followed by 10 s at 40 °C (cool down).

Calculation of Relative Telomere Length

We used the LinRegPCR software package (version 2016.0; (60)) to correct our amplification curves for baseline fluorescence, and to calculate well-specific reaction efficiencies and Cq values. A constant fluorescent threshold was set within the window of linearity for each amplicon group, calculated using the average Cq across all plates. The threshold values used were 0.2 and 0.25, and the average PCR efficiency across all plates was 1.876 and 1.881 for the B2M and telomere amplicon groups, respectively. Samples were excluded from further analysis if the coefficient of variation (CV) across triplicate Cq values and triplicate PCR efficiency values for either amplicon was > 5% (n=1; note that for > 95% of our samples the triplicate CV was < 2%), or if at least one of their triplicate reactions had an efficiency that was 5% higher or lower than the mean efficiency across all wells on that plate for the respective amplicon (n=2; > 95% of samples were within 2% of mean plate efficiencies; SI Appendix, Figure S4).

RLTL for each sample was calculated, following Pfaffl (61), using average reaction efficiencies for each plate and Cq for each sample determined by LinRegPCR as follows:

$$RTL = \frac{E_{TEL}^{(Cq_{TEL}[Calibrator] - Cq_{TEL}[Sample])}}{E_{B2M}^{(Cq_{B2M}[Calibrator] - Cq_{B2M}[Sample])}}$$

Where E_{TEL} and E_{B2M} are the mean reaction efficiencies for the respective amplicon group across all samples on a given plate; $Cq_{TEL}[Calibrator]$ and $Cq_{B2M}[Calibrator]$ are the average Cqs for the relevant amplicon across all calibrator samples on the plate; and $Cq_{TEL}[Sample]$ and $Cq_{B2M}[Sample]$ are the average of the triplicate Cqs for the sample for each amplicon.

Repeatability of telomere length

To assess the repeatability of our qPCR assay, the first qPCR plate (n=48 samples) was run 8 times over 4 consecutive days: four times over two days with samples in the same position

and then four times over two days with samples in an alternative row within the qPCR plate. We calculated the overall repeatability of RTL as the proportion of variance explained by sample identity over the total variance, in a linear mixed-effects model including only sample identity as a random intercept term (using restricted maximum likelihood estimation in *glmmTMB* v.0.2.3 (62)). The overall repeatability of RTL was 0.866 (95% confidence intervals 0.807–0.908). The repeatability of RTL measured in the same location across the first four qPCR plates was 0.948 (95%CI 0.922–0.965), illustrating how repeatability can be inflated if samples are consistently run in the same location. In a second model which included sample identity, qPCR plate and qPCR row as random intercept terms, the proportion of variance explained by qPCR plate and row was 0.005 and 0.063, respectively. The repeatability of RTL in this model was 0.824 (95%CI 0.748–0.880), or 0.884 (95%CI 0.829–0.923) if the plate and row terms were excluded from the total variance (since they represent measurement error). These repeatability estimates compare favourably with other studies of telomere length measured by both qPCR (inter-assay repeatability: 0.85 (48); 0.82 (24)) and terminal restriction fragment (TRF repeatability: 0.86 (63)).

Data analysis

All analyses were conducted in the program R version 3.6.1 (64) using the package *MCMCglmm* v.2.29 (65) unless otherwise specified.

Relationship between telomere length, age and sex

Relative telomere length was approximately normally distributed in lambs, adults and overall (Figure S5). We ran a series of linear mixed-effects models to determine the function that best described variation in telomere length with age. We included a two-level factor for age class (lamb: ~4 months of age; adult: ≥ 1 year). To account for age-related variation within the adult age class, we included age in years as a fixed covariate. We tested linear, quadratic and cubic age terms, as well as threshold age functions with a range of breakpoints (2–11 years). We also tested a four-level factor for age class (lamb: ~4 months of age; yearling: ~16 months; adult: 2-6 years; geriatric: >6 years. All models included individual identity and sample year as random intercept terms to account for non-independence among observations. The qPCR plate and row for each sample were also included as crossed random intercept terms to account for variation associated with measurement error. The models were run using maximum likelihood estimation in *glmmTMB* v.0.2.3 (62) and AIC model selection was used to determine the best age function. We selected the model with the fewest parameters within 2 ΔAIC of the model with the lowest AIC value. Once we determined the best fitting age function, we tested for a difference in average telomere length between the sexes by including a two-level factor for sex in our

model. To test whether the ageing patterns differed between the sexes, we also tested for interactions between sex and the selected age terms. The significance of additive and interactive effects of sex was assessed using likelihood ratio tests (see SI Appendix). The repeatability of telomere length over the lifespan was estimated as the variance explained by the random effect of individual over the total phenotypic variance (the sum of the random effects variance components plus the residual variance). There were 3641 observations of telomere length from 1586 sheep available for this analysis. Of these individuals, 836 had one RTL measurement available, 271 had two, 281 had three or four, and 198 had five our more measurements.

Heritability of telomere length

A quantitative genetic animal model was used to estimate the additive genetic variance for telomere length in Soay sheep. The model contained a two-level fixed factor for age class (lamb: ~4 months; adult: ≥ 1 year) and a linear covariate for age in years. We also included sex as a two-level fixed factor to account for differences in average telomere length between the sexes. The additive genetic effect was estimated using information on individual relatedness from the population pedigree. We included maternal identity in addition to the additive genetic component to capture similarity among maternal siblings that is not explained by the additive genetic effect (known as the maternal effect). We also included individual identity as a random effect to capture consistent differences in measures from the same individual that are not attributed to genetic effects, influenced for example by where the individual lives or aspects of their early life environment (the permanent environment effect). Year of sample collection, qPCR plate and qPCR row were included as random intercept terms. The heritability of telomere length was calculated as the variance explained by the additive genetic effect over the total phenotypic variance. This model was run for 1.1×10^5 iterations, with 1×10^4 burn-in and thinning interval of 50, resulting in 2000 stored samples of the MCMC chain with minimal autocorrelation (<0.2). Parameter estimates are presented as the posterior mode with 95% highest posterior density (HPD) intervals. Parameter expanded priors were used for all variance components, and inverse-Wishart priors for the residual variance. There were 3632 observations of telomere length from 1582 sheep available for this analysis (n=9 observations from 4 sheep were excluded because maternal identity was unknown).

We also ran a bivariate model to estimate the genetic correlation between telomere length in lambs (~4 months) and adults (≥ 1 year) which treated TL in lambs and adults as separate response variables. We included sex as a two-level fixed factor for both lambs and adults, with age in years included as a fixed covariate for adults only. qPCR plate and row were

included as random intercept terms across both models. Maternal identity was included as a random effect in the lamb model and individual identity in the adult model to estimate maternal and permanent environment effects, respectively. We estimated the unstructured variance-covariance matrix for the genetic, year and residual effects, which enabled us to estimate the correlations across age classes at these different hierarchical levels. There is a possibility that the lamb residual variance could covary with the adult permanent environment effect, but we did not attempt to model this covariance because we detected no permanent environment effect in adults (see Results). The model was run 2.1×10^5 iterations, with 1×10^4 burn-in and thinning interval of 200, resulting in 1000 stored samples of the MCMC chain (autocorrelation <0.2). The genetic correlation was taken from the posterior correlation of the stored samples with 95% HPD intervals. The bivariate model was run using 3632 measurements from 1582 individuals. Both lamb and adult telomere length measurements were available for 424 individuals.

Associations between telomere length, August weight and overwinter survival

Phenotypic model

We used a multivariate mixed-modelling approach to examine the association between telomere length, weight and overwinter survival at different hierarchical levels. Relative telomere length, body weight in kg (both measured August year *t*), and annual survival (to 1 May year *t+1*) were our response variables, modelled as Gaussian, Gaussian and threshold distributions, respectively (corresponding to identity and probit link functions). Unstructured variance–covariance matrices were estimated for some of the random effects, allowing us to estimate the covariance among the three traits at different hierarchical levels. For each of these random effects, we obtained a posterior distribution for the variance and covariance between telomere length ("RTL"), weight ("Wt") and overwinter survival ("Surv"):

$$\begin{bmatrix} \sigma^2_{RTL} & \sigma_{RTL,Wt} & \sigma_{RTL,Surv} \\ \sigma_{RTL,Wt} & \sigma^2_{Wt} & \sigma_{Wt,Surv} \\ \sigma_{RTL,Surv} & \sigma_{Wt,Surv} & \sigma^2_{Surv} \end{bmatrix}$$

We estimated the covariance at the among-individual, among-year and residual levels. The among-individual level captures consistent differences between individuals (e.g. if individuals that are consistently heavier tended to have longer lifespans). The covariance at the year level captures associations between average trait values among years (e.g. whether the survival rate is higher in years when the average body weight is higher). The residual level reflects covariance that is not captured by the among-individual and among-year levels (i.e. if an individual has a relatively low body weight compared to its average has lower chances of surviving).

The model was run for $5.3x10^5$ iterations, with $3x10^4$ burn-in and thinning interval of 500, resulting in 1000 stored samples of the MCMC chain (autocorrelation <0.1). The residual variance was fixed at 1 for survival (as is the standard recommendation for threshold models), and the latent variables were constrained to be between ±7 to avoid numerical difficulties as the probabilities approached 0 and 1. In addition to the random effects of identity and year, the telomere length model included random intercept terms for qPCR plate and row, and the weight and survival models included random intercept terms for maternal identity and birth year. We fitted age and sex in slightly different ways across the three models, to reflect our understanding of differences in age-related variation in these three traits. In the telomere model, we included age class (lamb vs adult) as a two-level fixed factor. No other fixed effects were included in the telomere model, since we were interested in how telomere length covaried with the other traits across the lifespan. In the weight model, we included age class (lamb vs adult) plus linear, quadratic and cubic age terms (capturing variation in weight with age during adulthood) and their interactions with sex. In a previous study, we detected a decline in weight across the year prior to death in this system using univariate models of weight (35). We did not include a term of final year alive here, as this effect is captured in the residual covariance between weight and survival in our multivariate model. In the survival model, we included age class (lamb vs adult) plus sex and its interaction with age class, as well as linear and quadratic age terms (capturing variation in survival with age during adulthood). This model was run using 3569 measurements from 1574 individuals for which telomere length, August weight and overwinter survival measurements were available. Telomere length and body weight were z-transformed prior to inclusion in the model (mean=0, standard deviation=1). To check the consistency of our results, we additionally ran separate models for lambs aged 4 months and adults aged ≥ 1 year (see Supplementary methods and results).

Genetic model

We extended our multivariate analysis to examine the association between telomere length, August body weight and overwinter survival probability at the genetic level (the G matrix). As with the univariate heritability analysis, we used information on individual relatedness from the population pedigree to estimate the additive genetic variances and covariances. In addition to the additive genetic (co)variance, we estimated the covariance at the residual, among-year and among-individual levels (the permanent environment covariance: the covariance among traits at the individual level that is not explained by genetic effects). The model was run for $5.3x10^5$ iterations, with $3x10^4$ burn-in and thinning interval of 500, resulting in 1000 stored samples of the MCMC chain (autocorrelation <0.1). Parameter expanded priors were used for all variance components, and inverse-Wishart priors for the

residual variances. The residual variance was fixed at 1 for survival (as is the standard recommendation for threshold models), and the latent variables were constrained to be between ±7 to avoid numerical difficulties as the probabilities approached 0 and 1. Other fixed and random effects were as stated above for the phenotypic models. We additionally ran separate animal models for lambs aged 4 months and adults aged ≥ 1 year to check the consistency of our results (see Supplementary methods and results).

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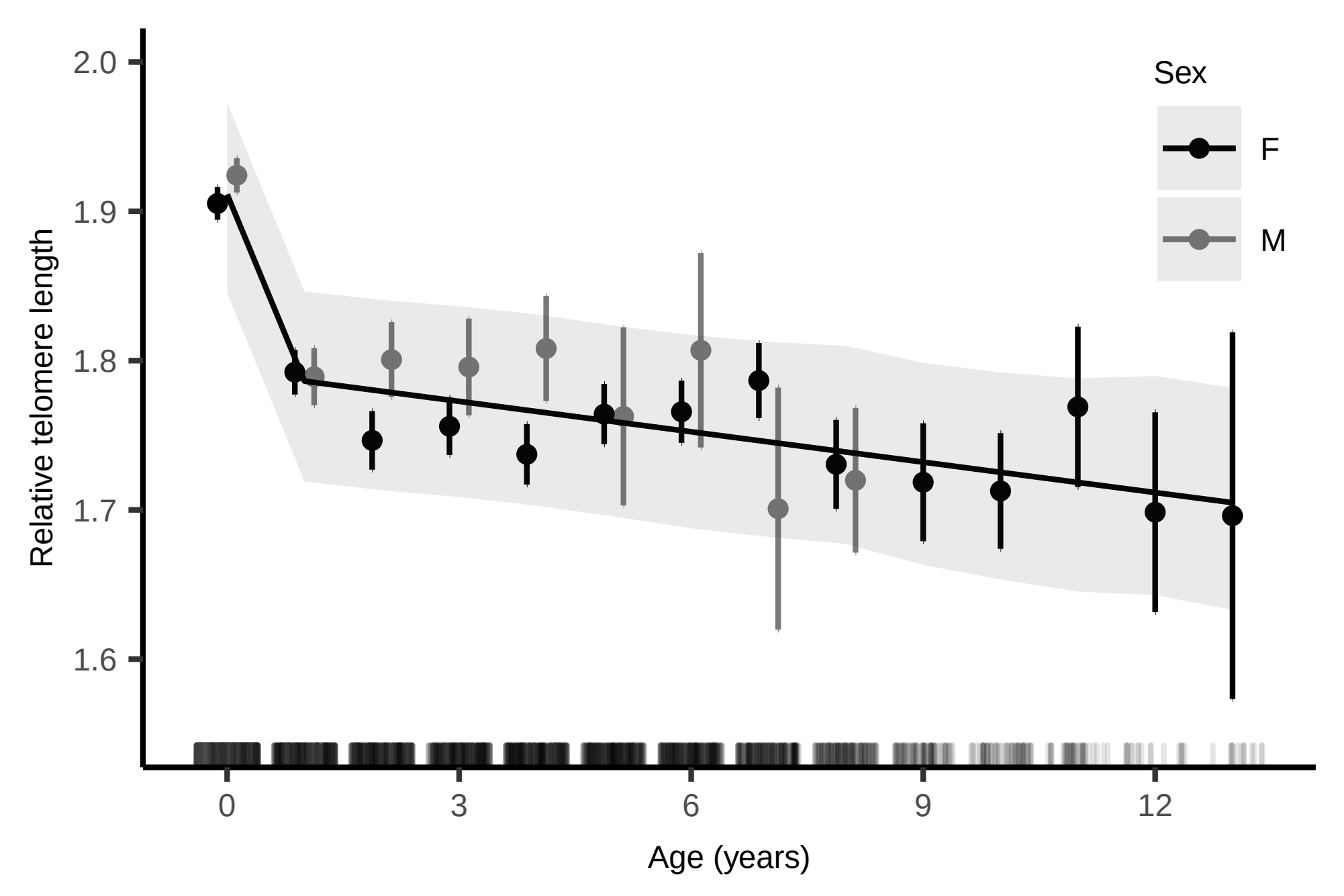
Figure legends

Figure 1. Relative telomere length varied with age over the lifespan in Soay sheep (n=3641 observations of 1586 individuals). The points show raw data medians and standard errors for each age, with females in black and males in grey (for clarity, n=3 observations of females aged > 13 and n=6 observations of males aged > 8 are grouped with ages 13 and 8, respectively). The black lines show predictions from the best model (SI Appendix, Table S2), grey shading represents 95% credible intervals around those predictions. The rug plot on the inside of the x-axis shows the distribution of observations across the age range. Note that the ageing pattern in the raw data and best-fitting age functions from mixed-effects models are not expected to align perfectly when selective disappearance effects are present.

Figure 2. The proportion of variance in relative telomere length in Soay sheep explained by different variance components. Estimates are based on the mode of the posterior distribution from Bayesian quantitative genetic animal models. Estimates for age class 'all' came from a univariate model incorporating all observations (n=3632 of 1582 individuals) that included sex, age class and age in years as fixed effects (SI Appendix, Table S3). The separate estimates for adults (age ≥ 1 year) and lambs (4 months old) came from a bivariate model that accounted for the covariance between lamb and adult telomere length at the genetic, year and residual levels (SI Appendix, Table S4).

Figure 3. The correlation between relative telomere length (RTL), August body weight and overwinter survival probability at different hierarchical levels in Soay sheep (n=3569 observations of 1574 individuals). Correlations were estimated as the mode of the posterior distribution with 95% higher probability density intervals from multivariate Bayesian mixed-effects models. Panel A shows estimates for the among-individual (teal), among-year (black) and residual correlations (grey) from a phenotypic model (SI Appendix, Table S5). Panel B shows estimates for the genetic (teal circles), permanent environment ("PermEnv", teal triangles), among-year (black) and residual correlations (grey) from a quantitative genetic animal model (SI Appendix, Table S6).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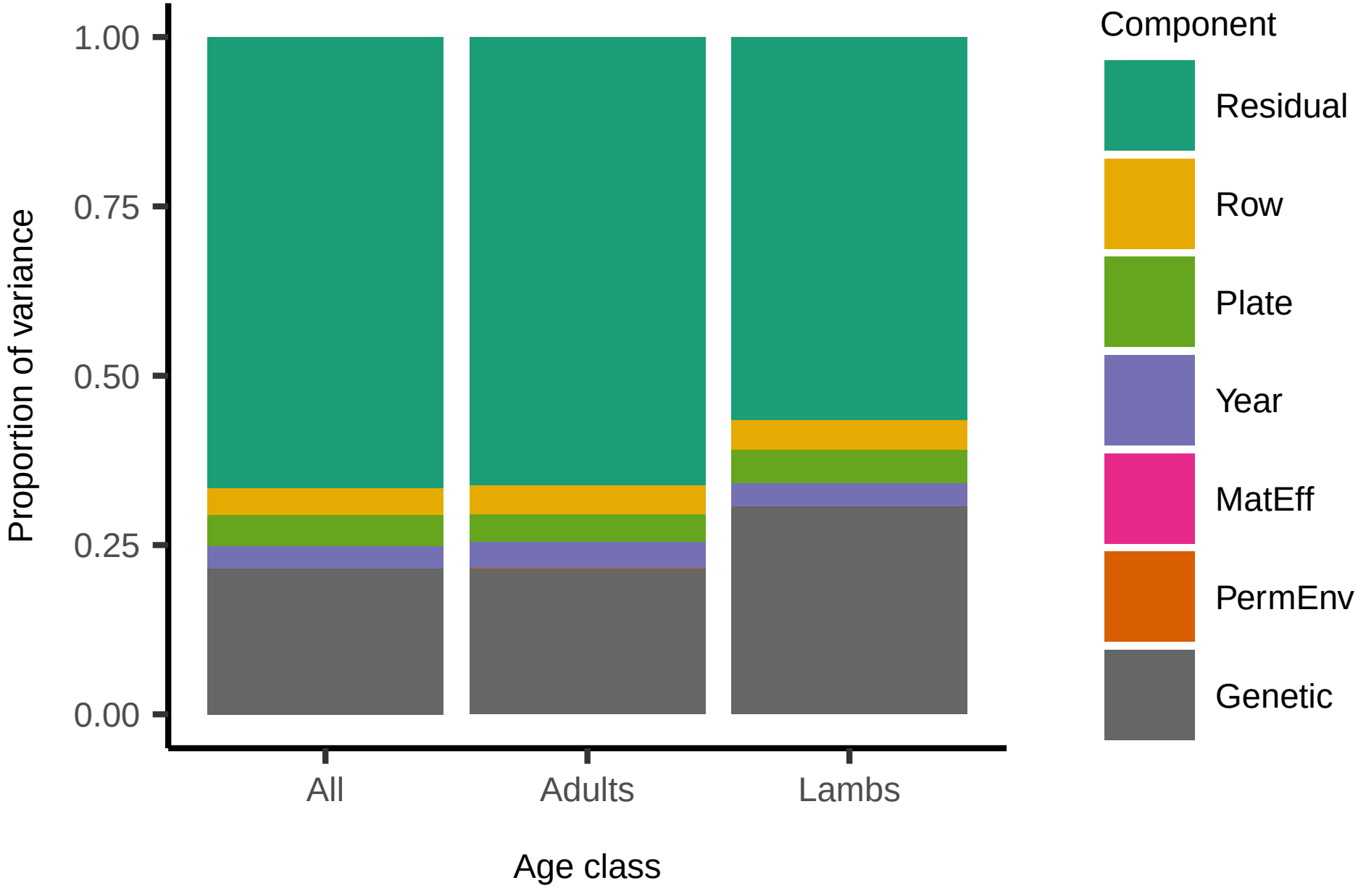
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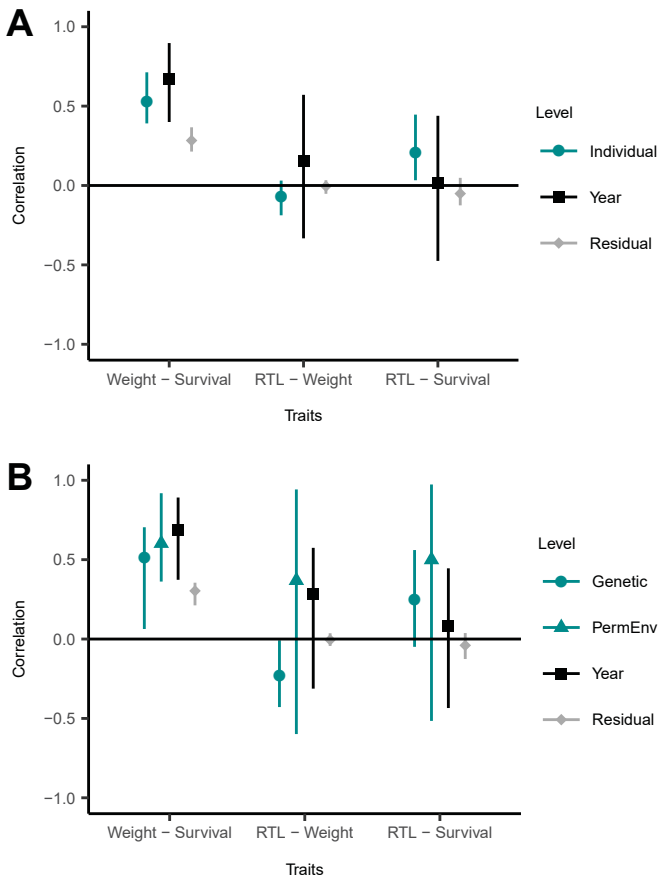
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